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# REVERSIBLE HYPOTHERMIA-INDUCED INHIBITION OF HUMAN PLATELET ACTIVATION IN WHOLE BLOOD IN VITRO AND IN VIVO

BY

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Platelets and other blood components are often transfused in clinical settings associated with hypothermia and a bleeding diathesis, such as cardiopulmonary bypass surgery, other major surgery, and multiple trauma. We examined the hypothesis that hypothermia reversibly inhibits human platelet activation in vitro and in vivo. Methods: Platelet activation was studied in normal human volunteers by whole blood flow cytometric analysis of modulation of platelet surface GMP-140 and the glycoprotein (GP)

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Ib-IX complex in a) shed blood emerging from a standardized in vivo bleeding time wound and b) peripheral blood activated in vitro with either x-thrombin (in the presence of gly-pro-arg-pro, an inhibitor of fibrin polymerization) or the stable thromboxane A2 analogue U46619. Platelets in peripheral whole blood were activated at temperatures between 22 C and 37 C. The skin temperature of the forearm was maintained at temperatures between 22 C and 37 C prior to and during the bleeding time incision. Platelet aggregation was studied in shed blood by flow cytometry using a gate on both GPIb-positivity and light scatter and in peripheral blood by aggregometry. Results: In vitro, hypothermia inhibited the rate and extent of both thrombin and U46619-induced a) upregulation of GMP-140, b) downregulation of the GPIb-IX complex, c) platelet aggregation, d) platelet shape change, and e) thromboxane B2 generation. These inhibitory effects of hypothermia were all completely reversed by rewarming the blood to 37 C. In vivo, platelet activation was inhibited by hypothermia as shown by 5 independent assays of shed blood: 1) upregulation of GMP-140, 2) downregulation of the GPIb-IX complex, 3) platelet aggregate formation, 4) thromboxane B2 generation, and 5) the bleeding time. Summary: By a combination of immunological, biochemical, and functional assays, we have demonstrated that hypothermia inhibits human platelet activation in whole blood in vitro and in vivo. Rewarming hypothermic blood completely reverses the activation defect. These results suggest that rewarming a hypothermic bleeding patient can reduce the need for the less safe and more expensive alternative of transfusion of platelets and other blood components.

## ABSTRACT

Platelets and other blood components are often transfused in clinical settings associated with hypothermia and a bleeding diathesis, such as cardiopulmonary bypass surgery, other major surgery, and multiple trauma. We examined the hypothesis that hypothermia reversibly inhibits human platelet activation *in vitro* and *in vivo*. ***Methods:*** Platelet activation was studied in normal human volunteers by whole blood flow cytometric analysis of modulation of platelet surface GMP-140 and the glycoprotein (GP) Ib-IX complex in a) shed blood emerging from a standardized *in vivo* bleeding time wound and b) peripheral blood activated *in vitro* with either $\alpha$-thrombin (in the presence of gly-pro-arg-pro, an inhibitor of fibrin polymerization) or the stable thromboxane $A_2$ analogue U46619. Platelets in peripheral whole blood were activated at temperatures between 22°C and 37°C. The skin temperature of the forearm was maintained at temperatures between 22°C and 37°C prior to and during the bleeding time incision. Platelet aggregation was studied in shed blood by flow cytometry using a gate on both GPIb-positivity and light scatter and in peripheral blood by aggregometry. ***Results:*** *In vitro*, hypothermia inhibited the rate and extent of both thrombin- and U46619-induced a) upregulation of GMP-140, b) downregulation of the GPIb-IX complex, c) platelet aggregation, d) platelet shape change, and e) thromboxane $B_2$ generation. These inhibitory effects of hypothermia were all completely reversed by rewarming the blood to 37°C. *In vivo*, platelet activation was inhibited by hypothermia as shown by 5 independent assays of shed blood: 1) upregulation of GMP-140, 2) downregulation of the GPIb-IX complex, 3) platelet aggregate formation, 4) thromboxane $B_2$ generation, and 5) the bleeding time. ***Summary:*** By a combination of immunological, biochemi-

cal, and functional assays, we have demonstrated that hypothermia inhibits human platelet activation in whole blood *in vitro* and *in vivo*. Rewarming hypothermic blood completely reverses the activation defect. These results suggest that rewarming a hypothermic bleeding patient can reduce the need for the less safe and more expensive alternative of transfusion of platelets and other blood components.

# INTRODUCTION

Hypothermia occurs frequently in a number of clinical settings, including hypothermic cardiopulmonary bypass surgery, other major surgery, multiple trauma, exposure, and neonatal cold injury. A hypothermia-induced hemorrhagic diathesis is associated with these clinical settings (1-7). However, the pathophysiological basis of this hemorrhagic diathesis is not well understood. Hypothermia has been reported to induce a platelet function defect in baboons, as evidenced by diminished generation of thromboxane $B_2$ (8) and prolongation of the bleeding time (2,8). Hypothermia also results in prolongation of the bleeding time in humans (9). However, the specificity of the relationship between the bleeding time and platelet function has been challenged (10).

In the present study, we tested the hypothesis that hypothermia inhibits human platelet activation in whole blood *in vitro* and *in vivo*. We utilized the following combination of immunological, biochemical, and functional assays of platelet activation: upregulation of the platelet surface expression of GMP-140 (reflecting $\alpha$ granule secretion) (11), downregulation of the platelet surface expression of the GPIb-IX complex (the von Willebrand factor receptor) (12), thromboxane $B_2$ generation (the stable metabolite of thromboxane $A_2$) (13), platelet aggregate formation (14), and the bleeding time. Because platelets and other blood components are often transfused in clinical settings associated with hypothermia, we also examined the possibility that the safer and less expensive measure of rewarming could reverse the putative hypothermia-induced platelet activation defect.

# METHODS

## *Murine Monoclonal Antibodies*

S12 (provided by Dr. Rodger P. McEver, University of Oklahoma) is directed against GMP-140 (15). GMP-140, also referred to as platelet activation-dependent granule-external membrane (PADGEM) protein (16), CD62 (17), and P-selectin (18), is a component of the $\alpha$ granule membrane of resting platelets that is only expressed on the platelet plasma membrane after platelet secretion (11). 6D1 (provided by Dr. Barry S. Coller, SUNY, Stony Brook) is directed against the von Willebrand factor receptor on the glycocalicin portion of the $\alpha$-chain of platelet membrane glycoprotein (GP) Ib (19,20). AK3 (provided by Dr. Michael C. Berndt, University of Sydney, Australia) is directed against the macroglycopeptide portion of the $\alpha$ chain of platelet membrane GPIb (21). FMC25 and AK1 (both provided by Dr. Berndt) are directed against platelet membrane GPIX (22,23) and the platelet membrane GPIb-IX complex (24), respectively. AK1 only binds to the intact GPIb-IX complex, not to uncomplexed GPIb or GPIX (24). Y2/51 (DAKO, Carpinteria, CA) is directed against platelet membrane GPIIIa (25).

## *Flow Cytometric Analysis of Platelets in Peripheral Whole Blood*

The method has been previously described (26). Blood was drawn by venipuncture from healthy adult volunteers who had not ingested aspirin within the previous 10 days. The first 2 ml of blood were discarded and then blood was drawn directly into a sodium citrate Vacutainer (Becton Dickinson, Rutherford, NJ). Prior to activation, the blood, purified human $\alpha$-thrombin (provided by Dr. John W. Fenton II, New York Department of Health, Albany), the peptide

glycyl-L-prolyl-L-arginyl-L-proline (GPRP) (Calbiochem, San Diego, CA), the thromboxane $A_2$ analogue U46619 (Cayman Chemical, Ann Arbor, MI), and modified Tyrode's buffer (137 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM $Na_2HPO_4$, 0.35% bovine serum albumin, 10 mM HEPES, 5.5 mM glucose, pH 7.4) were equilibrated to one of the following temperatures: 22°C, 32°C, 37°C. At each of these temperatures, the blood was then incubated with one of the following: a) $\alpha$-thrombin 5 U/ml and (to prevent fibrin polymerization (26)) GPRP 2.5 mM; b) U46619 10 $\mu$M; or c) buffer only. Platelet activation was stopped at various time points by the addition of 1% formaldehyde to each aliquot. After fixation for 30 minutes at 22°C, the samples were diluted 10-fold in the modified Tyrode's buffer.

In experiments designed to study the reversibility of hypothermia-induced changes, the method was as above, except that the diluted whole blood (500 $\mu$L) was initially incubated at 37°C for 5 minutes and an aliquot was activated with thrombin, then the same blood was cooled to 22°C over 15 minutes and an aliquot activated, and finally the same blood was rewarmed to 37°C over 5 minutes and an aliquot activated. In these experiments, the thrombin was diluted into multiple aliquots on ice and then individual aliquots were equilibrated to the activation temperature.

Platelets in the fixed diluted whole blood samples were labeled with fluorescently conjugated murine monoclonal antibodies as previously described (26). The samples were then analyzed in an EPICS Profile flow cytometer (Coulter Cytometry, Hialeah, FL) for activation-dependent changes in the platelet binding of phycoerythrin-streptavidin/biotin-conjugated antibodies S12 (GMP-140-specific) and 6D1 (GPIb-specific), as previously described (26). Fluorescein isothiocyanate (FITC)-conjugated antibody Y2/51 (GPIIIa-specific) was used as the

platelet-specific marker (26). The experimental conditions (diluted whole blood, no stirring, GPRP together with $\alpha$-thrombin) allowed us to study single platelets in whole blood without inducing the formation of platelet aggregates (26).

***Flow Cytometric Analysis of Platelets and Platelet Aggregates in Whole Blood Emerging from a Bleeding Time Wound***

The method of analysis of platelets has been previously described (26). The study was approved by the Institutional Review Boards of the University of Massachusetts Medical School and Boston University School of Medicine. Healthy adult volunteers who were taking no medications participated in the study after providing written informed consent. The forearm skin temperature of volunteers was equilibrated to between 22°C and 37°C by one or more of the following methods: a stream of air cooled by dry ice or wet ice, a walk-in 4°C cold room, a hair dryer, a heating lamp. Local skin temperature was monitored by a surface thermometer (Skin Temperature Sensor, Mon-A-Therm, St. Louis, MO) placed within a few millimeters of the bleeding time incision. Duplicate standardized bleeding times were performed with a Simplate II device (General Diagnostics, Durham, NC) as previously described (27). The blood emerging from the bleeding time wound was collected with a micropipet at 2 minute intervals until the bleeding stopped. After each pipetting, any residual blood at the bleeding time wound site was removed with filter paper. Immediately after collection at each time point, the pipetted blood (1 $\mu$L per antibody tested) was added to a microfuge tube containing sodium citrate, fixed for 30 minutes at 22°C with an equal volume of 2% formaldehyde, and diluted 1:10 by volume in modified Tyrode's buffer. As described above, the fixed diluted whole blood samples were

then labeled with a FITC-conjugated GPIIIa-specific monoclonal antibody (Y2/51) and either a biotinylated GMP-140-specific monoclonal antibody (S12) or a biotinylated GPIb-IX complex-specific monoclonal antibody (6D1, AK3, FMC25, or AK1), and the individual platelets analyzed in an EPICS Profile flow cytometer for activation-dependent changes. To analyze the number of platelet aggregates in the blood emerging from the bleeding time wound, the fixed diluted whole blood was labeled with both FITC-conjugated monoclonal antibody Y2/51 (GPIIIa-specific) and phycoerythrin-streptavidin/biotin-conjugated monoclonal antibody 6D1 (GPIb-specific), and the samples analyzed by flow cytometry as described in the **Results** section.

***Platelet Aggregometry***

Platelets were washed as previously described (28) and resuspended at 250,000/$\mu$L in modified Tyrode's buffer, pH 7.4. The platelets were initially incubated at 37°C for 10 minutes, then the same platelets were cooled to 22°C over 30 minutes, and finally the same platelets were rewarmed to 37°C over 15 minutes. Prior to the activation step, the agonists were diluted into multiple aliquots on ice and individual aliquots were equilibrated to the activation temperature. At each incubation temperature, an aliquot of platelets was removed and incubated with either purified human $\alpha$-thrombin (final concentration 0.06 - 1 U/ml) or U46619 (final concentration 0.12 - 0.37 $\mu$M) in a platelet aggregometer (Lumi-Aggregation Module Series 10008, Payton, Buffalo, NY) that had been equilibrated to the same temperature as the platelets. The aggregometer was equilibrated to 22°C by turning off its heater and fanning of ice-cold air. The area under the aggregation curve at the 5 minute time point was measured by

a Hewlett-Packard 9874A Digitizer (Palo Alto, CA) and a Hewlett-Packard 9854N computer and expressed as digitizer units/5 minutes.

### *Radioimmunoassay of Supernatant Thromboxane $B_2$ In Vitro*

In the platelet aggregometry experiments described above, generation of thromboxane $B_2$ was stopped after 5 minutes by the addition of 1 mg/mL ibuprofen (Upjohn, Kalamazoo, MI). The samples were immediately placed on ice and centrifuged (1,650 g, 10 minutes, 4°C). The supernatants were stored at -80°C until assayed for thromboxane $B_2$ with an RIA kit (New England Nuclear, Boston, MA), as previously described (8).

### *Radioimmunoassay of Shed Blood Thromboxane $B_2$*

After the skin temperature of the forearm of a normal volunteer was equilibrated to either 22°C, 28°C, or 32°C, a standardized bleeding time wound was performed, as described above. Shed blood in 600 $\mu$L aliquots was collected continuously from the beginning to the end of the bleeding time. Each aliquot was assayed for thromboxane $B_2$ with an RIA kit (New England Nuclear), as previously described (8). The rate of thromboxane $B_2$ generation (pg/0.1 mL/second) was calculated from the total thromboxane $B_2$ generation from all aliquots divided by the bleeding time in seconds.

### *Data Analysis*

Results are expressed as mean $\pm$ standard error of the mean (S.E.M.) unless otherwise stated. Statistical analysis was performed by Student's t-test for paired or independent samples,

as appropriate, or by one way analysis of variance (ANOVA). Differences were considered statistically significant if p values were less than 0.05.

# RESULTS

## *Effect of Temperature on Activation-induced Changes in Platelet Surface GMP-140 and GPIb In Vitro*

We first examined the effect of hypothermia on thrombin-induced changes in platelet surface expression of GMP-140 and GPIb *in vitro* (Fig 1). To avoid the possibility of artefactual platelet activation *in vitro*, a whole blood flow cytometric method was used (26). This method did not result in any artefactual platelet activation, as demonstrated by the complete lack of binding of the GMP-140-specific monoclonal antibody S12 in the absence of added exogenous thrombin (Fig 1, panel A, 0 time point). As previously described (26), at 37°C thrombin resulted in upregulation of the platelet surface expression of GMP-140 (reflecting $\alpha$ granule secretion) (Fig 1, panel A) and, after a delay of approximately 20 seconds, downregulation of the platelet surface expression of GPIb (the von Willebrand factor receptor) (Fig 1, panel B). As the temperature was reduced from 37°C to 22°C, the thrombin-induced upregulation of the platelet surface expression of GMP-140 (Fig 1, panel A) and the thrombin-induced downregulation of the platelet surface expression of GPIb (Fig 1, panel B) proceeded more slowly. However, the final extent of the upregulation of GMP-140 and the downregulation of GPIb was the same irrespective of temperature (Fig 1, panels A and B).

We then examined the effect of the stable thromboxane $A_2$ analogue U46619 on platelet surface expression of GMP-140 and GPIb. At 37°C, U46619 resulted in upregulation of the platelet surface expression of GMP-140 to almost the same extent as thrombin (Fig 2, panel A). At 37°C, U46619 resulted in downregulation of the platelet surface expression of GPIb, although

not to the same extent as thrombin (Fig 2, panel B). Analogous to the situation with thrombin (Fig 1), as the temperature was reduced from 37°C to 22°C, the U46619-induced upregulation of the platelet surface expression of GMP-140 (Fig 2, panel A) and the U46619-induced downregulation of the platelet surface expression of GPIb (Fig 2, panel B) proceeded more slowly. Even after 10 minutes incubation of the blood with U46619 at 22°C, the extent of upregulation of GMP-140 and of downregulation of GPIb did not achieve that at 32° and 37°C (Fig 2, panels A and B).

To compare directly thrombin- and U46619-induced platelet activation at the earliest (and presumably the most physiologically important) time points, data from Figs 1 and 2 were replotted as shown in Figs 3 and 4. At all temperatures tested (37°C, 32°C, and 22°C), the kinetics of the upregulation of GMP-140 and the downregulation of GPIb were more rapid with thrombin than with U46619 (Figs 3 and 4). Within the first 1½ minutes at 22°C, platelets were only minimally activated in response to U46619, whereas they did respond to thrombin, albeit at a slower rate than normal (Fig 3, panel C and Fig 4, panel C).

***Reversibility of Hypothermia-induced Inhibition of Platelet Activation***

To address the question as to whether the hypothermia-induced inhibition of platelet activation was reversible, 4 independent assays were utilized:

***1) Upregulation of Platelet Surface GMP-140***

Diluted whole blood was incubated at 37°C, cooled to 22°C, and then the same whole blood sample was rewarmed to 37°C (Fig 5). As determined by the whole blood flow cytometric assay, the hypothermia-induced slowing of the kinetics of thrombin-induced

upregulation of platelet surface GMP-140 (Fig 5, open circles: compare panel A to panel B) was completely reversed by rewarming of the blood to 37°C (Fig 5, open circles: panel C).

*2) Downregulation of the Platelet Surface GPIb-IX complex*

In the same experiment as in 1) above, the hypothermia-induced slowing of the kinetics of thrombin-induced downregulation of GPIb (Fig 5, closed circles: compare panel A to panel B) was completely reversed by rewarming of the blood to 37°C (Fig 5, closed circles: panel C), as determined by the whole blood flow cytometric assay.

*3) Platelet Aggregation*

An important functional indication of platelet activation is platelet aggregate formation (14). Washed platelets were incubated at 37°C, cooled to 22°C, and then the same platelets were rewarmed to 37°C. Both thrombin- and U46619-induced platelet aggregation was inhibited at 22°C, as determined by shape change, lag phase, slope, and maximal amplitude (Fig 6, compare panel A to panel B and panel D to panel E). At 22°C there was an almost complete absence of shape change (Fig 6, panels B and E). The hypothermia-induced inhibition of platelet aggregation was completely reversed by rewarming of the blood to 37°C, as determined by shape change, lag phase, slope, and maximal amplitude (Fig 6, panels C and F). Analysis of the area under the platelet aggregation curve 5 minutes after activation with 0.24 $\mu$M U46619 resulted in the following digitizer units (as defined in **Methods**): 188.0 $\pm$ 6.1 at 37°C, 128.0 $\pm$ 18.5 at 22°C, and 189.3 $\pm$ 4.2 after rewarming to 37°C (mean $\pm$ S.E.M., n = 3). Similar results were obtained when the area under the thrombin-induced aggregation curve was analyzed (Fig 7: panels A and B).

*4) Thromboxane $B_2$ Generation*

Generation of thromboxane $A_2$ is another important marker of platelet activation (13). In the same experiment as in 3) above, generation of thromboxane $B_2$ (the stable metabolite of thromboxane $A_2$) was assayed in the supernatants 5 minutes after thrombin-induced platelet activation (Fig 7: panels C and D). As shown for thrombin 0.4 U/mL (Fig 7: panel C) and thrombin 0.16 U/mL (Fig 7: panel D), thromboxane $B_2$ generation was inhibited at 22°C, but rewarming to 37°C resulted in complete reversal of this inhibition. Similar results were obtained with thrombin 1 U/mL (data not shown).

*Effect of Temperature on Platelet Activation In Vivo*

To address the question as to whether hypothermia-induced inhibition of platelet activation can occur *in vivo*, the skin temperature of the forearms of normal volunteers was equilibrated to temperatures between 22°C and 37°C. The shed blood emerging from a standardized bleeding time wound was then analyzed by 5 independent assays of platelet activation:

*1) The Bleeding Time*

The bleeding time was markedly prolonged by hypothermia (Fig 8). Reduction of the skin temperature from 37°C to 22°C resulted in prolongation of the bleeding time from 5.0 $\pm$ 0.4 to 23.3 $\pm$ 3.4 minutes (mean $\pm$ S.E.M., n = 6) (Fig 8).

*2) Upregulation of Platelet Surface GMP-140*

Shed blood was analyzed for the normal time-dependent upregulation of platelet surface GMP-140 (26,29) (Fig 9, panel A). The time-dependent upregulation of GMP-

140 was abolished by hypothermia (22°C) (Fig 9: panel A). Again, as indicated by the end points of the solid lines, the bleeding time was prolonged by hypothermia (Fig 9: panels A, B, and C).

### *3) Downregulation of the Platelet Surface GPIb-IX Complex*

Shed blood was analyzed for the normal time-dependent downregulation of the platelet surface GPIb-IX complex (26) (Fig 9, panels B and C). The time-dependent downregulation of the GPIb-IX complex was almost abolished by hypothermia (22°C), as determined by a GPIb-specific monoclonal antibody (6D1) (Fig 9, panel B) and a GPIb-IX complex-specific monoclonal antibody (AK1) (Fig 9, panel C). Parallel results were obtained with a monoclonal antibody (AK3) directed against a different epitope on GPIb and a monoclonal antibody (FMC25) directed against GPIX (data not shown).

### *4) Platelet Aggregate Formation*

To analyze the number of platelet aggregates in the blood emerging from the bleeding time wound, the fixed diluted whole blood was labeled and analyzed by flow cytometry as described in Fig 10. Reduction of the forearm skin temperature of normal volunteers resulted in a reduction in both the rate of formation and the maximal number of platelet aggregates emerging from a standardized bleeding time wound (Fig 11).

### *5) Thromboxane $B_2$ Generation*

Reduction of the forearm skin temperature of normal volunteers resulted in a reduction of both the rate of generation and the maximal concentration of thromboxane $B_2$ in the blood emerging from a standardized bleeding time wound (Fig 12). The rate of

thromboxane $B_2$ generation (pg/0.1 mL/second) was 2.1 $\pm$ 0.7 at 32°C, 0.75 $\pm$ 0.2 at 28°C, and 0.09 $\pm$ 0.03 at 22°C (mean $\pm$ S.E.M., n = 6) ($p < 0.05$ by ANOVA).

### *The Effects of Temperature Were Not Confined to a Subpopulation of Platelets*

Because each platelet is analyzed individually, the flow cytometric method of analyzing platelet surface glycoproteins is able to detect distinct subpopulations of platelets (30,31). However, in all the experiments described above, the effects of hypothermia were evident on all the platelets, rather than confined to a distinct subpopulation of platelets (data not shown).

### *Effect of Gender on Temperature-dependent Changes in Platelet Activation*

The temperature-dependent changes in platelet activation *in vitro* and *in vivo* were not significantly different between male and female volunteers (data not shown).

# DISCUSSION

Although a hypothermia-induced hemorrhagic diathesis is known to occur in a number of clinical settings, including hypothermic cardiopulmonary bypass surgery, other major surgery, multiple trauma, exposure, and neonatal cold injury (1-7), the pathophysiological basis of this hemorrhagic diathesis is not well understood. The hemorrhagic diathesis associated with hypothermic cardiopulmonary bypass surgery is considered to be primarily a platelet function defect (1,2,32). We have recently demonstrated that hypothermia correlates with both prolongation of the bleeding time and postoperative blood loss in patients undergoing cardiopulmonary bypass surgery (3,33). However, this clinical setting is complex because even during normothermic cardiopulmonary bypass surgery there is a platelet function defect (2). We (8) and others (2) have demonstrated that hypothermia results in prolongation of the bleeding time in normal baboons. We also recently reported that hypothermia prolongs the bleeding time in normal human volunteers (9). However, the specificity of the relationship between the bleeding time and platelet function has been challenged (10).

In the present study, we established that hypothermia inhibits human platelet activation in whole blood *in vivo*, as determined in normal volunteers by five independent assays of shed blood emerging from a standardized bleeding time wound: 1) upregulation of platelet surface GMP-140 (reflecting $\alpha$ granule secretion), 2) downregulation of the platelet surface GPIb-IX complex (the von Willebrand factor receptor), 3) platelet aggregate formation, 4) thromboxane $B_2$ generation, and 5) the bleeding time.

In a previous study in baboons (8), we reported that the pathophysiological basis for hypothermia-induced platelet dysfunction was, at least in part, inhibition of the action of thromboxane synthetase, resulting in diminished generation of thromboxane $A_2$. In the present study in humans, we have confirmed and expanded upon these observations. Thus, hypothermia resulted in lack of generation of thromboxane $B_2$ (the stable metabolite of thromboxane $A_2$) in humans both *in vitro* and *in vivo*. In addition, we now demonstrate that hypothermia inhibits human platelet activation in whole blood *in vitro*, as determined by $\alpha$-thrombin- and U46619-induced: a) upregulation of platelet surface GMP-140, b) downregulation of the platelet surface GPIb-IX complex, and c) platelet aggregation and shape change. Thus, in addition to their lack of generation of thromboxane $A_2$, hypothermic platelets are less reactive to thromboxane $A_2$ (as determined by the stable analogue U46619), as well as to thrombin.

In this study, we also demonstrated that rewarming hypothermic blood completely reverses the activation defect, as determined by four independent methods: upregulation of platelet surface GMP-140, downregulation of the platelet surface GPIb-IX complex, platelet aggregation, and thromboxane $B_2$ generation. The reversibility of the hypothermia-induced inhibition of platelet activation suggests that rewarming a hypothermic bleeding patient can reduce the need for transfusions of platelets and other blood components. Rewarming of the patient should be considered prior to transfusion given the risks (34) and expense of blood transfusion. Furthermore, once a hypothermia-induced hemorrhagic diathesis develops, transfusion of platelets or plasma is relatively ineffective (5). The present data suggest that rewarming the patient will result in better function of transfused platelets. Our data also suggest

that every effort should be made to maintain normothermia in patients predisposed to hypothermia, such as during major surgery, multiple trauma, and the neonatal period.

The whole blood flow cytometric assay utilized in this study has many advantages over other assays of platelet activation, such as plasma assays of ß-thromboglobulin and platelet factor 4 (35), and should be applicable to many other clinical settings. The assay enabled us to study: 1) human $\alpha$-thrombin (the preeminent platelet activator *in vivo* (36-38)) in the physiological milieu of whole blood, through the use of GPRP, an inhibitor of fibrin polymerization (26); 2) platelet activation *in vivo* at the site of a standardized bleeding time wound, because only about 1 $\mu$L of blood is required for the assay (26,29); 3) platelet aggregate formation *in vivo* (this study); and, 4) activation-induced upregulation of the platelet surface expression of GMP-140 (11) and downregulation of the platelet surface expression of the GPIb-IX complex (12) on individual platelets (26). In addition, artefactual *in vitro* platelet activation as a result of experimental manipulation, a major problem in assays such as ß-thromboglobulin and platelet factor 4 (35), was avoided (26,39).

In summary, by a combination of immunological, biochemical, and functional assays, we have demonstrated that hypothermia inhibits human platelet activation in whole blood both *in vitro* and *in vivo*. Rewarming hypothermic blood completely reverses the activation defect. These results suggest that rewarming a hypothermic bleeding patient can reduce the need for the less safe and more expensive alternative of transfusion of platelets and other blood components.

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## FIGURE LEGENDS

**Fig 1.** Effect of hypothermia on thrombin-induced modulation of the expression of GMP-140 and GPIb on the surface of individual platelets in whole blood *in vitro*. Purified human α-thrombin 5 U/ml and GPRP 2.5 mM (to prevent fibrin polymerization) were added at the 0 time point to whole blood incubated at either 37°C, 32°C, or 22°C. Platelet activation was stopped at various time points by the addition of 1% formaldehyde. The platelet surface binding of monoclonal antibodies S12 (GMP-140-specific) (panel A) and 6D1 (GPIb-specific) (panel B) was analyzed by whole blood flow cytometry, as described in **Methods**. Panel A: maximum GMP-140 was defined as the binding of S12 after incubation with thrombin at 37°C for 15 minutes. Panel B: maximum GPIb was defined as the binding of 6D1 in the absence of thrombin. The experimental conditions (diluted whole blood, no stirring, GPRP together with α-thrombin) permitted the study of single platelets in whole blood without inducing the formation of platelet aggregates. Data from panels A and B were obtained from aliquots of the same samples. Data are mean $\pm$ S.E.M., $n = 4$ separate experiments. Asterisks indicate $p < 0.05$ by paired Student's t test, as compared to samples at 37°C.

**Fig 2.** Effect of hypothermia on the modulation of platelet surface GMP-140 and GPIb induced by the thromboxane $A_2$ analogue U46619 in whole blood *in vitro*. U46619 10 $\mu$M was added at the 0 time point to whole blood incubated at either 37°C, 32°C, or 22°C. In parallel experiments, thrombin 5 U/ml and GPRP 2.5 mM were added at the 0 time point to whole blood incubated at 37°C. Platelet activation was stopped at various time points by the addition of 1% formaldehyde. The platelet surface binding of monoclonal antibodies S12 (GMP-140-specific) (panel A) and 6D1 (GPIb-specific) (panel B) was analyzed by whole blood flow cytometry. Panel A: maximum GMP-140 was defined as the binding of S12 after incubation with thrombin at 37°C for 15 minutes. Panel B: maximum GPIb was defined as the binding of 6D1 in the absence of agonist. Data from panels A and B were obtained from aliquots of the same samples. Data are mean $\pm$ S.E.M., n = 3 separate experiments. Asterisks indicate $p < 0.05$ by paired Student's t test, as compared to samples at 37°C.

**Fig 3.** Direct comparison of the initial kinetics of platelet surface exposure of GMP-140 induced by thrombin (open circles) and U46619 (closed circles) in whole blood *in vitro*. These data are from the same experiments shown in Fig 1, panel A and Fig 2, panel A. Thrombin 5 U/ml and GPRP 2.5 mM or U46619 10$\mu$M alone were added at the 0 time point to whole blood incubated at either 37°C (panel A), 32°C (panel B), or 22°C (panel C). Platelet activation was stopped at various time points by the addition of 1% formaldehyde. Platelet surface GMP-140 was determined by whole blood flow cytometry as in Figs 1 and 2. Maximum GMP-140 was defined as in Figs 1 and 2. Asterisks indicate that the thrombin and U46619 samples were significantly different from each other ($p < 0.05$ by independent Student's t test).

**Fig 4.** Direct comparison of the initial kinetics of the downregulation of platelet surface GPIb induced by thrombin (open circles) and U46619 (closed circles) in whole blood *in vitro*. These data are from the same experiments shown in Fig 1, panel B and Fig 2, panel B. Thrombin 5 U/ml and GPRP 2.5 mM or U46619 10 $\mu$M alone were added at the 0 time point to whole blood incubated at either 37°C (panel A), 32°C (panel B), or 22°C (panel C). Platelet activation was stopped at various time points by the addition of 1% formaldehyde. Platelet surface GPIb was determined by whole blood flow cytometry as in Figs 1 and 2. Maximum GPIb was defined as in Figs 1 and 2. Asterisks indicate that the thrombin and U46619 samples were significantly different from each other ($p < 0.05$ by independent Student's t test).

**Fig 5.** Reversibility of hypothermia-induced inhibition of platelet activation, as determined by whole blood flow cytometric analysis of platelet surface GMP-140 and GPIb. The experiment was performed as in Fig 1, except that the blood was initially incubated at 37°C for 5 minutes, then the same blood was cooled to 22°C over 15 minutes, and finally the same blood was rewarmed to 37°C over 5 minutes. At each incubation temperature, an aliquot of whole blood was removed and activated at the 0 time point with thrombin 5 U/ml in the presence of GPRP 2.5 mM (both of which had been equilibrated to the temperature of the blood). Maximum GMP-140 was defined as the binding of S12 after incubation with thrombin for 15 minutes at the first 37°C incubation. Maximum GPIb was defined as the binding of 6D1 in the absence of thrombin. Data are mean $\pm$ S.E.M., n = 3 separate experiments.

**Fig 6.** Reversibility of hypothermia-induced inhibition of platelet activation, as determined by platelet aggregation. Washed platelets 250,000/$\mu$L in modified Tyrode's buffer, pH 7.4 were initially incubated at 37°C for 10 minutes, then the same platelets were cooled to 22°C over 30 minutes, and finally the same platelets were rewarmed to 37°C over 15 minutes. At each incubation temperature, an aliquot of platelets was activated with either thrombin 0.16 U/ml or U46619 0.24 $\mu$M in a platelet aggregometer that had been equilibrated to the same temperature as the platelets and agonists. "Lag": time from addition of agonist (arrow) to start of increase in light transmission. "Slope": maximal change in light transmission per minute x 100. "Max": light transmission at 5 minutes. The experiment is representative of 7 so performed. Similar results were obtained in experiments with thrombin 0.06, 0.4, and 1 U/ml and with U46619 0.12 and 0.37 $\mu$M (data not shown).

**Fig 7.** Reversibility of hypothermia-induced inhibition of platelet activation, as determined by platelet aggregation and thromboxane $B_2$ generation. The experiment was the same as that shown in Fig 6. Platelet aggregation (panels A and B) is expressed as digitizer units/5 minutes, derived from the area under the aggregation curve as described in **Methods.** Generation of thromboxane $B_2$ was stopped after 5 minutes by the addition of 1 mg/mL ibuprofen. The samples were immediately placed on ice and centrifuged (1,650 g, 10 minutes, 4°C). The supernatants were assayed for thromboxane $B_2$ by radioimmunoassay (panels C and D). Panels A and C: thrombin 0.4 U/mL. Panel B and D: thrombin 0.16 U/mL. Data are mean $\pm$ S.E.M., n = 4 separate experiments. Asterisks indicate $p < 0.05$ by paired Student's t test, as compared to initial samples at 37°C.

**Fig 8.** Effect of skin temperature on a standardized bleeding time *in vivo*. The skin temperature of the forearms of healthy volunteers who were taking no medications were equilibrated to 22°C, 28°C, 32°C or 37°C by one or more of the following methods: a stream of air cooled by dry ice or wet ice, a walk-in 4°C cold room, a hair dryer, a heating lamp. Local skin temperature was monitored by a surface thermometer placed within a few millimeters of the bleeding time incision. Duplicate standardized bleeding times were performed with a Simplate II device. For the calculation of each data point the bleeding time was considered to be the mean of 2 duplicate bleeding times. Data are mean $\pm$ S.E.M., $n = 6$. Asterisks indicate $p < 0.05$ by paired Student's t test, as compared to skin temperature of 32°C (normothermia).

**Fig 9.** Effect of temperature on activation-induced modulation of platelet surface GMP-140 and the GPIb-IX complex in whole blood *in vivo*. After the skin temperature of the forearm of a normal volunteer was equilibrated to either 22°C, 32°C, or 37°C, a standardized bleeding time wound was performed as in Fig 8. The blood emerging from the bleeding time wound was fixed at 2 minute intervals until the bleeding stopped, as described in **Methods**. The fixed samples were then analyzed by whole blood flow cytometry for the platelet surface binding of monoclonal antibodies S12 (GMP-140-specific) (panel A), 6D1 (GPIb-specific) (panel B), and AK1 (GPIb-IX complex-specific) (panel C). Panel A: maximum GMP-140 was defined as the binding of S12 to platelets in peripheral blood after stimulation with thrombin 5 U/ml in the presence of GPRP 2.5 mM. Panels B and C: maximum GPIb was defined as the binding of 6D1 and AK1 to platelets in peripheral blood. Each panel shows data from the same experiment on the same donor. At each time point for each temperature, the determination of platelet surface GMP-140, GPIb, and the GPIb-IX complex was obtained from the same sample. The experiment is representative of 5 so performed.

**Fig 10.** Flow cytometric method of analysis of platelet aggregates in whole blood emerging from a standardized bleeding time wound. Whole blood emerging from the bleeding time wound, as well as a peripheral blood sample obtained in parallel, was fixed, diluted, and labeled with both FITC-conjugated monoclonal antibody Y2/51 (GPIIIa-specific) and phycoerythrin-streptavidin/biotin-conjugated monoclonal antibody 6D1 (GPIb-specific), as previously described (26). Platelets and platelet aggregates were first discriminated from red cells and white cells by gating on positivity for Y2/51. A histogram of log forward light scatter (reflecting platelet size) versus 6D1 binding was then generated (panels A and B). In the peripheral whole blood samples, analysis regions were plotted around the 6D1-positive particles to include 95% of the population on the forward light scatter axis (panel A, region 1) and the 5% of particles appearing above this forward light scatter threshold (panel A, region 2). Identical regions were used for the whole blood samples obtained from the bleeding time wounds, as shown in panel B (forearm skin temperature 32°C, 6 minute time point). The number of platelet aggregates as a percentage of the number of single platelets was calculated from the following arbitrary formula: [(number of particles in region 2 of shed blood) - (number of particles in region 2 of peripheral blood)] ÷ (number of particles in region 1 of shed blood) x 100%.

**Fig 11.** Effect of temperature on platelet aggregate formation *in vivo*. After the skin temperature of the forearm of a single normal volunteer was equilibrated to either 22°C, 27°C, or 32°C, a standardized bleeding time wound was performed as in Fig 8. The blood emerging from the bleeding time wound was fixed at 2 minute intervals until the bleeding stopped. The number of platelet aggregates in whole blood was determined as in Fig 10. The experiment is representative of 5 so performed.

**Fig 12.** Effect of temperature on thromboxane $B_2$ generation in response to a standardized vascular injury *in vivo*. After the skin temperature of the forearm of a normal volunteer was equilibrated to either 22°C, 28°C, or 32°C, a standardized bleeding time wound was performed as in Fig 8. Shed blood in 600 $\mu$L aliquots was collected continuously from the beginning to the end of the bleeding time. At each temperature, the first and last aliquots were assayed for thromboxane $B_2$. Data are mean $\pm$ S.E.M., n = 6 separate experiments.

Fig 1

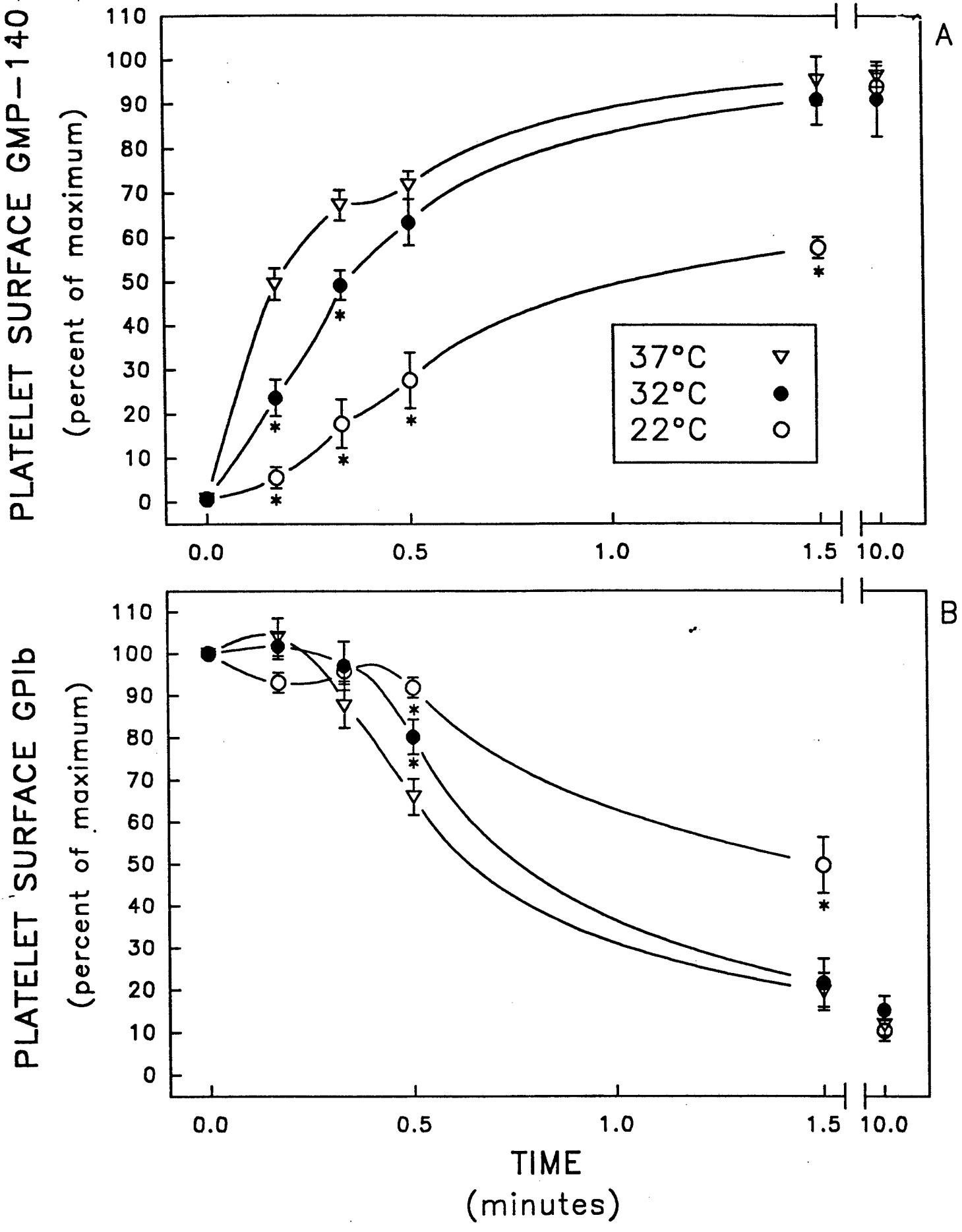

PLATELET SURFACE GMP-140
(percent of maximum)
110
100
90
80
70
60
50
40
30
20
10
0
0.0
0.5
1.0
1.5
10.0
A
37°C
32°C
22°C
PLATELET SURFACE GPIb
(percent of maximum)
B
TIME
(minutes)

Fig 2

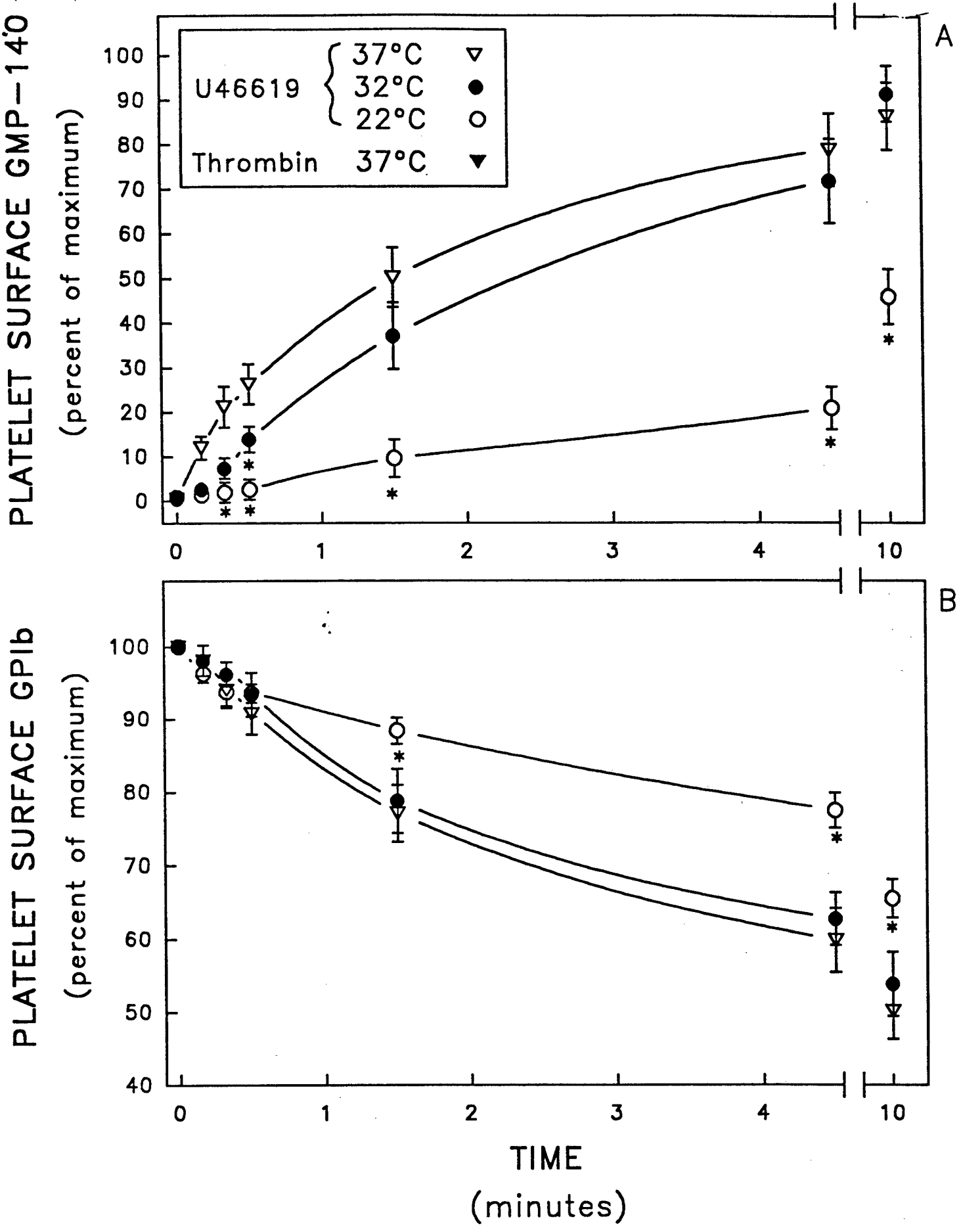

PLATELET SURFACE GMP-140
(percent of maximum)
U46619
37°C
32°C
22°C
Thrombin 37°C
A
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
10
PLATELET SURFACE GPIb
(percent of maximum)
B
100
90
80
70
60
50
40
0
1
2
3
4
10
TIME
(minutes)

Fig 3

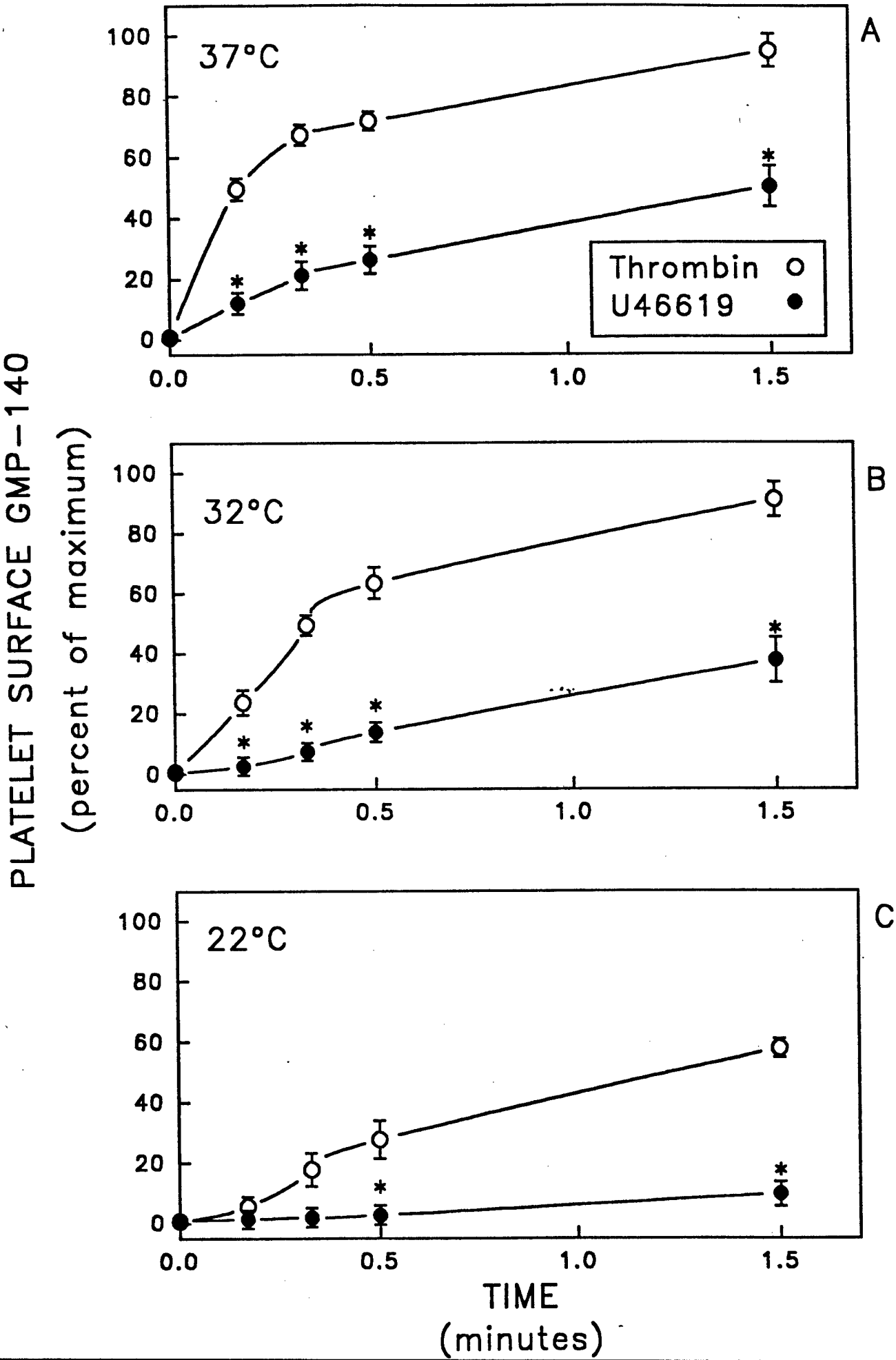

A
37°C
Thrombin
U46619
B
32°C
C
22°C
100
80
60
40
20
0
0.0
0.5
1.0
1.5
PLATELET SURFACE GMP-140
(percent of maximum)
TIME
(minutes)

Fig 4

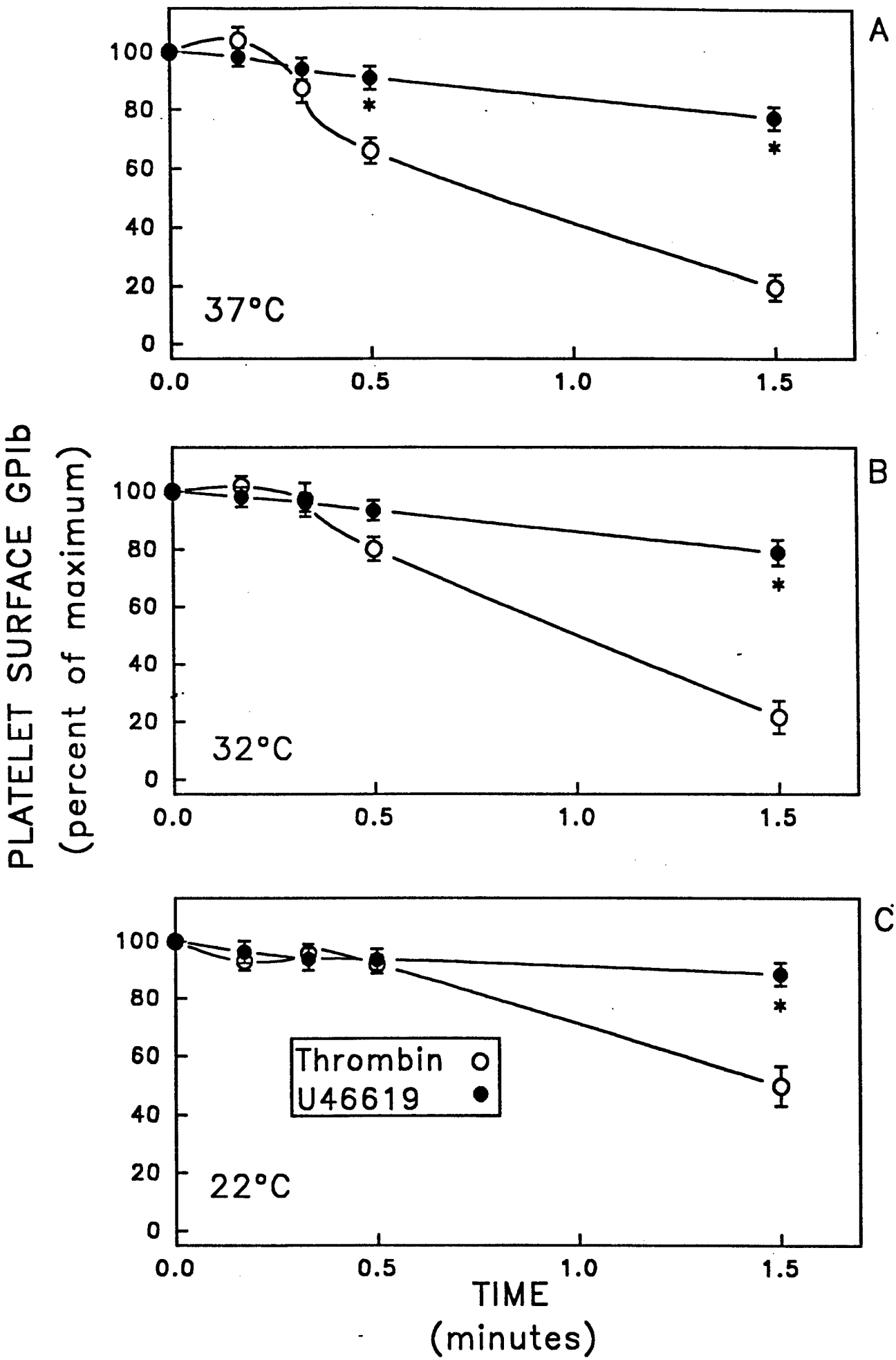

A
B
C
100
80
60
40
20
0
0.0
0.5
1.0
1.5
37°C
32°C
22°C
*
PLATELET SURFACE GPIb
(percent of maximum)
Thrombin
U46619
TIME
(minutes)

Fig 5

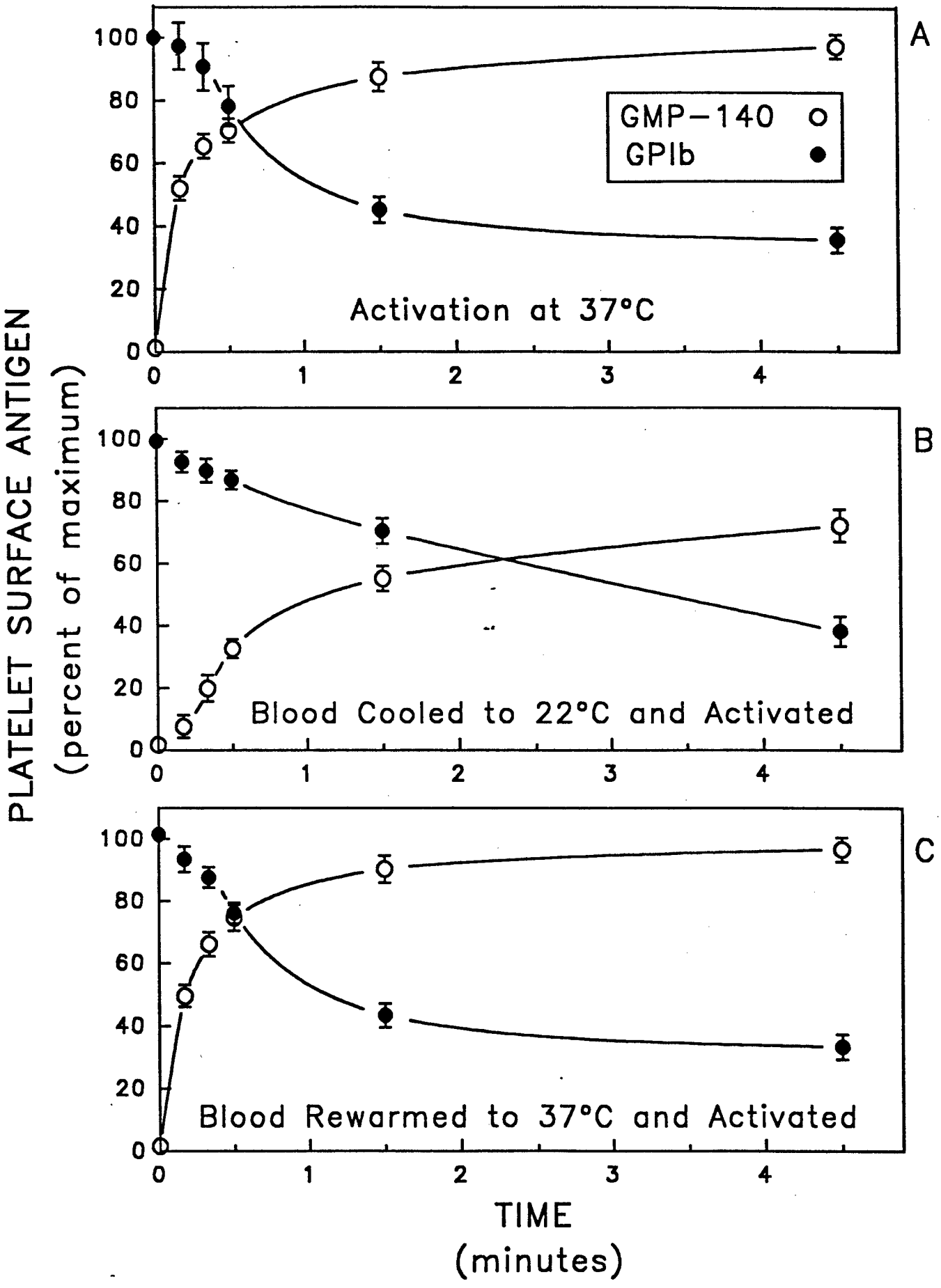
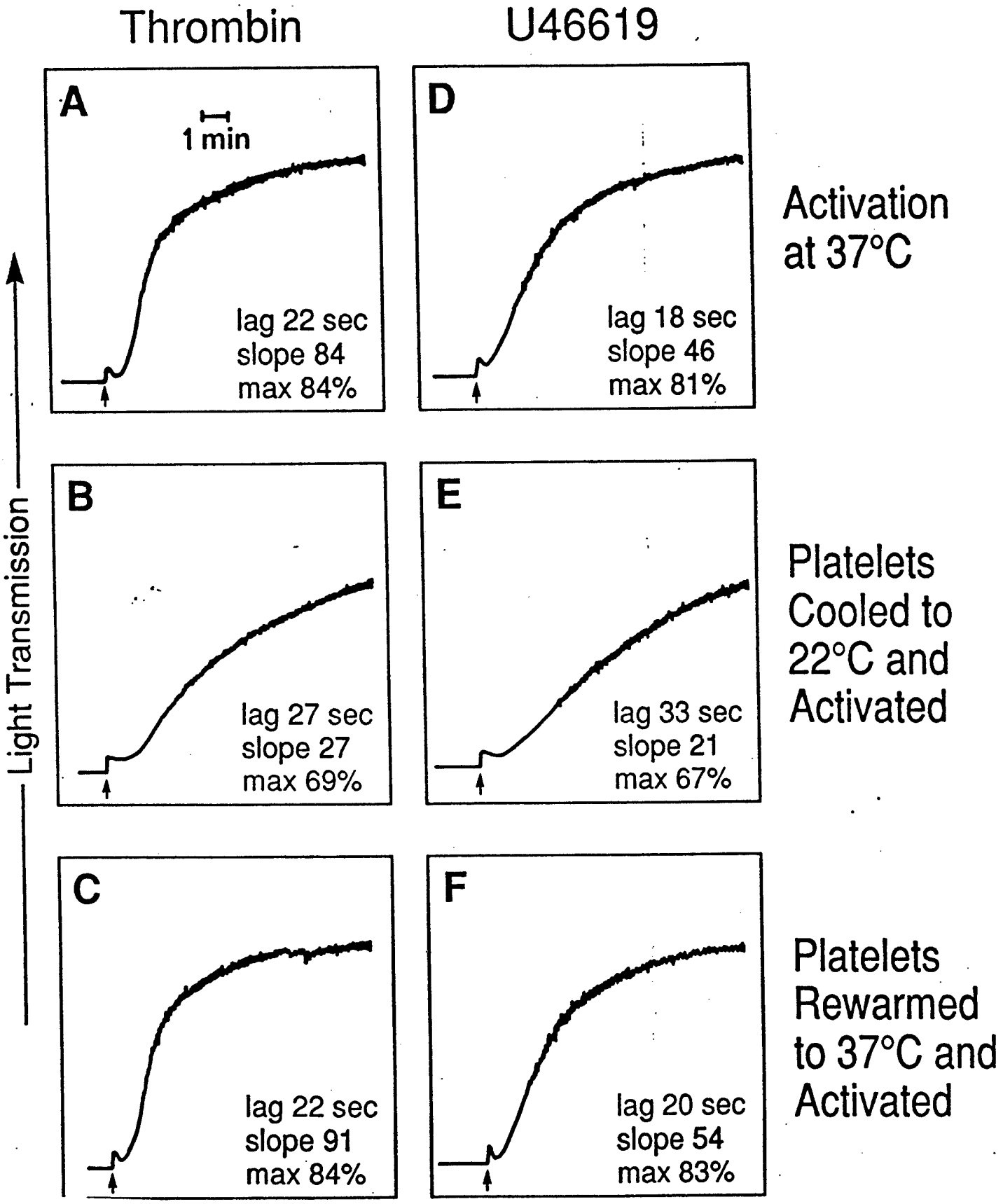

A
B
C
GMP–140
GPIb
Activation at 37°C
Blood Cooled to 22°C and Activated
Blood Rewarmed to 37°C and Activated
PLATELET SURFACE ANTIGEN
(percent of maximum)
TIME
(minutes)
0
20
40
60
80
100
0
1
2
3
4

Fig. 6


Thrombin
U46619
A
1 min
lag 22 sec
slope 84
max 84%
D
lag 18 sec
slope 46
max 81%
Activation at 37°C
B
lag 27 sec
slope 27
max 69%
E
lag 33 sec
slope 21
max 67%
Platelets Cooled to 22°C and Activated
C
lag 22 sec
slope 91
max 84%
F
lag 20 sec
slope 54
max 83%
Platelets Rewarmed to 37°C and Activated
Light Transmission

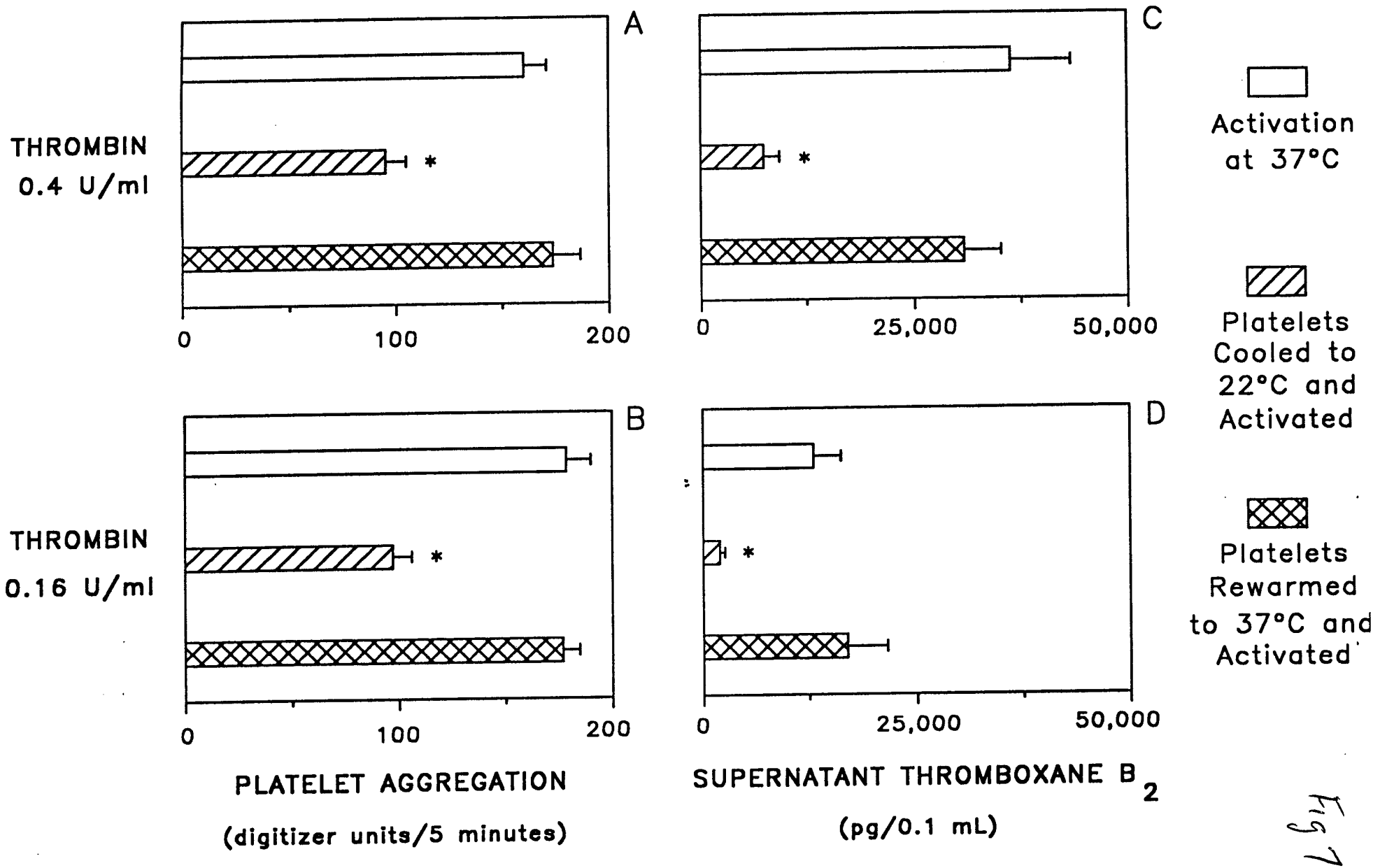

A
C
B
D
THROMBIN
0.4 U/ml
THROMBIN
0.16 U/ml
*
0
100
200
25,000
50,000
Activation at 37°C
Platelets Cooled to 22°C and Activated
Platelets Rewarmed to 37°C and Activated
PLATELET AGGREGATION
(digitizer units/5 minutes)
SUPERNATANT THROMBOXANE B2
(pg/0.1 mL)

Fig 7

Fig 8

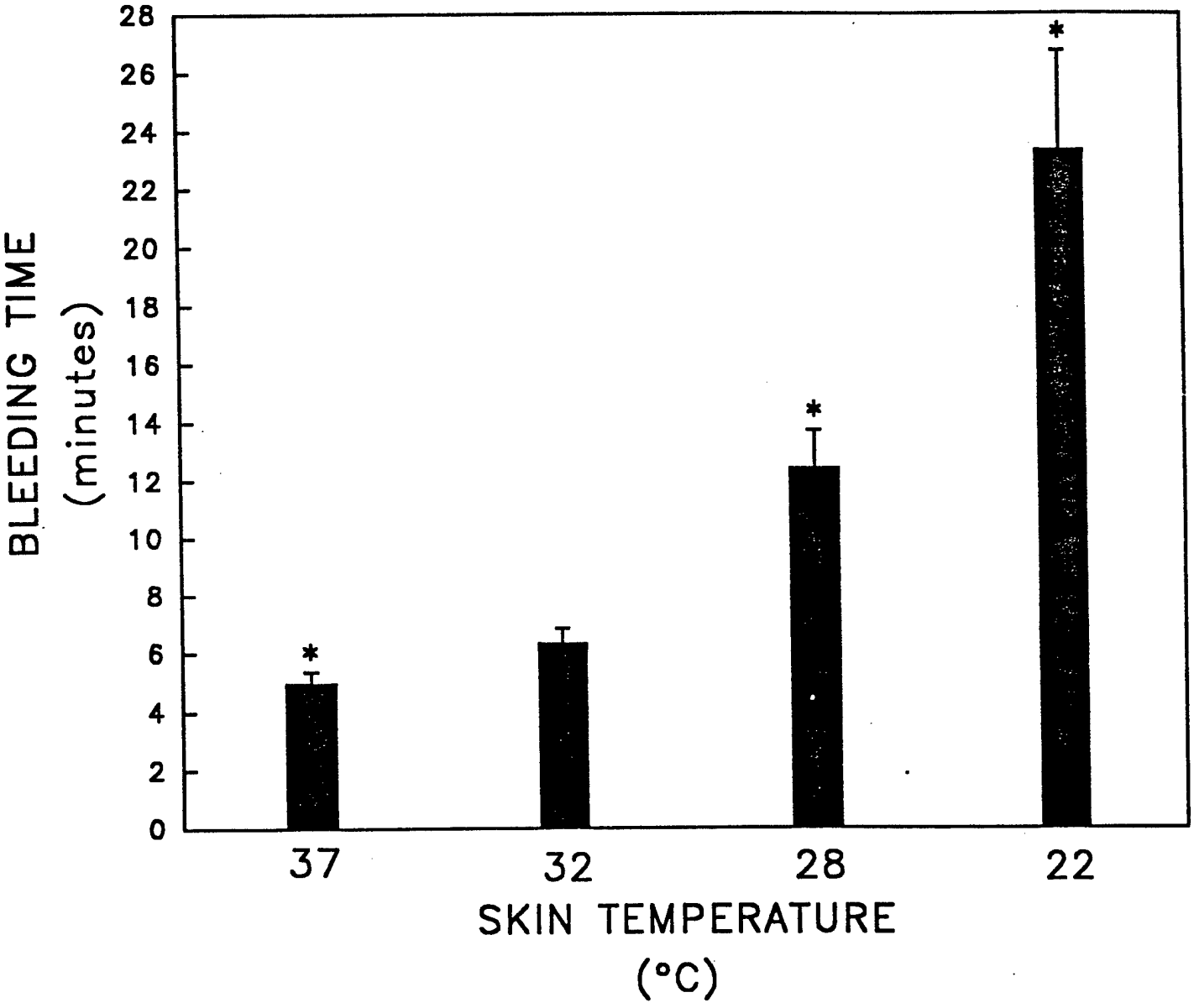

BLEEDING TIME
(minutes)
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
*
*
*
37
32
28
22
SKIN TEMPERATURE
(°C)

Fig. 9

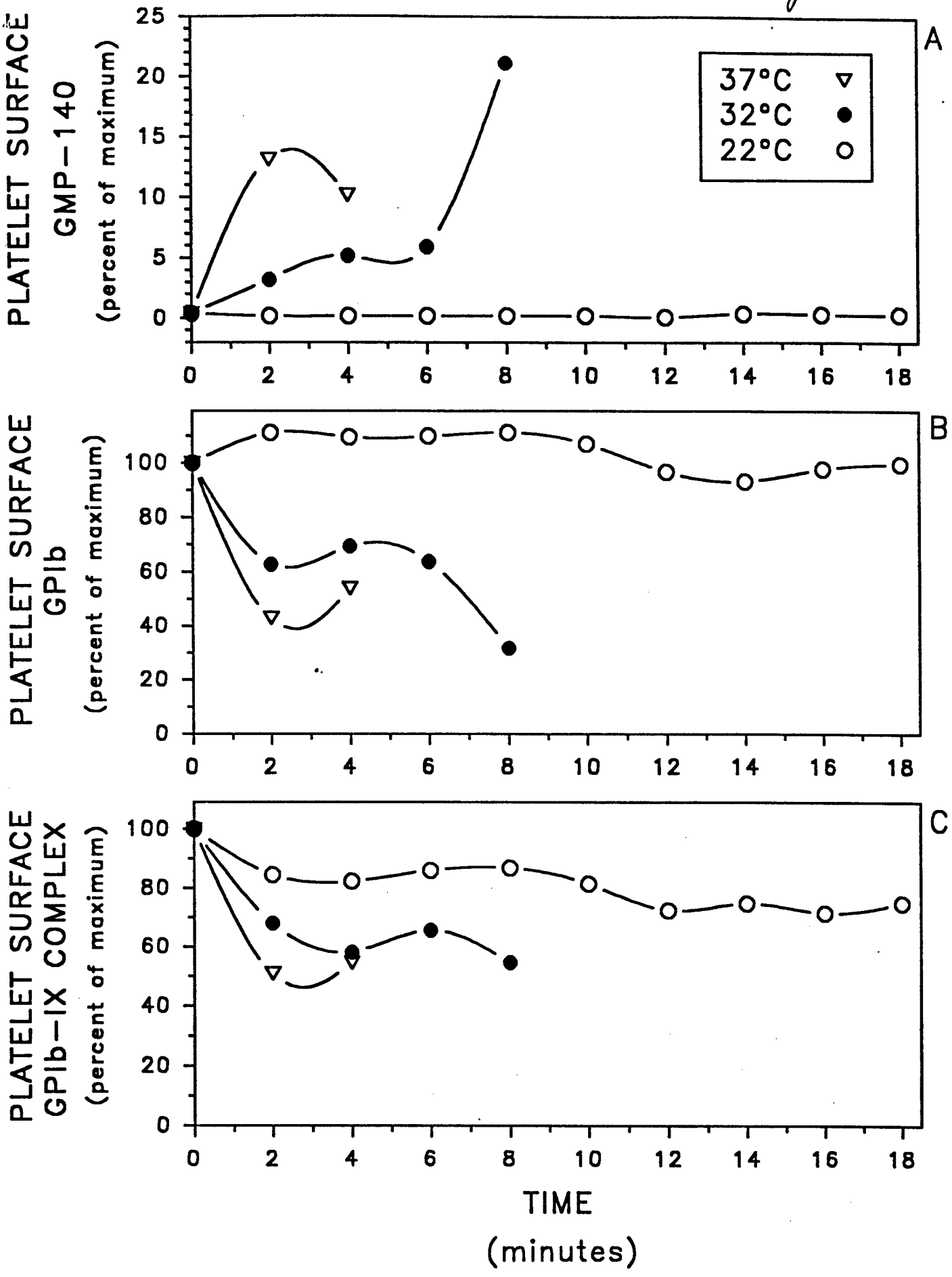

PLATELET SURFACE
GMP–140
(percent of maximum)
25
20
15
10
5
0
37°C
32°C
22°C
A
0
2
4
6
8
10
12
14
16
18
PLATELET SURFACE
GPIb
(percent of maximum)
100
80
60
40
20
0
B
PLATELET SURFACE
GPIb–IX COMPLEX
(percent of maximum)
C
TIME
(minutes)

Fig 10

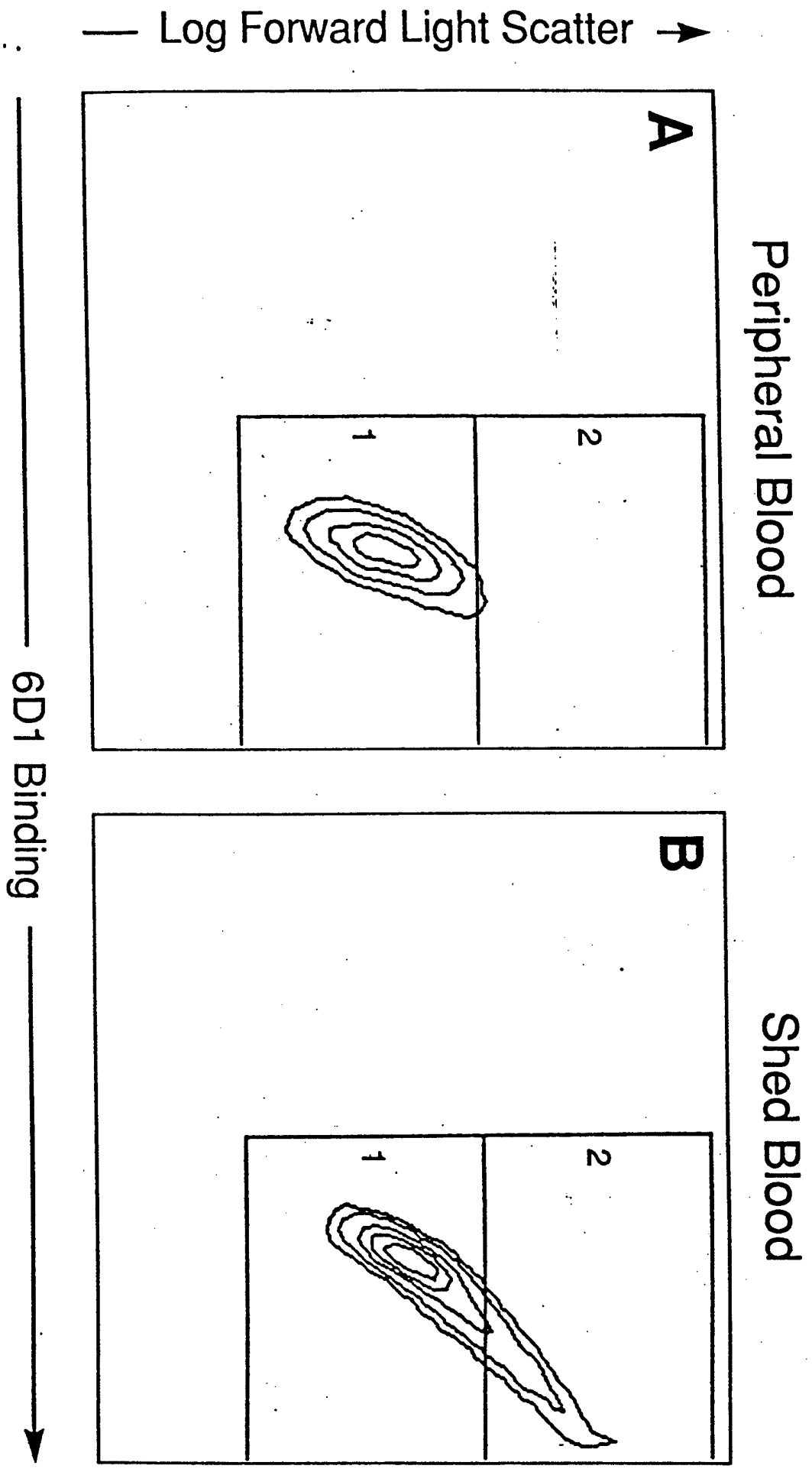

Log Forward Light Scatter
6D1 Binding
A
Peripheral Blood
1
2
B
Shed Blood
1
2

Fig 11

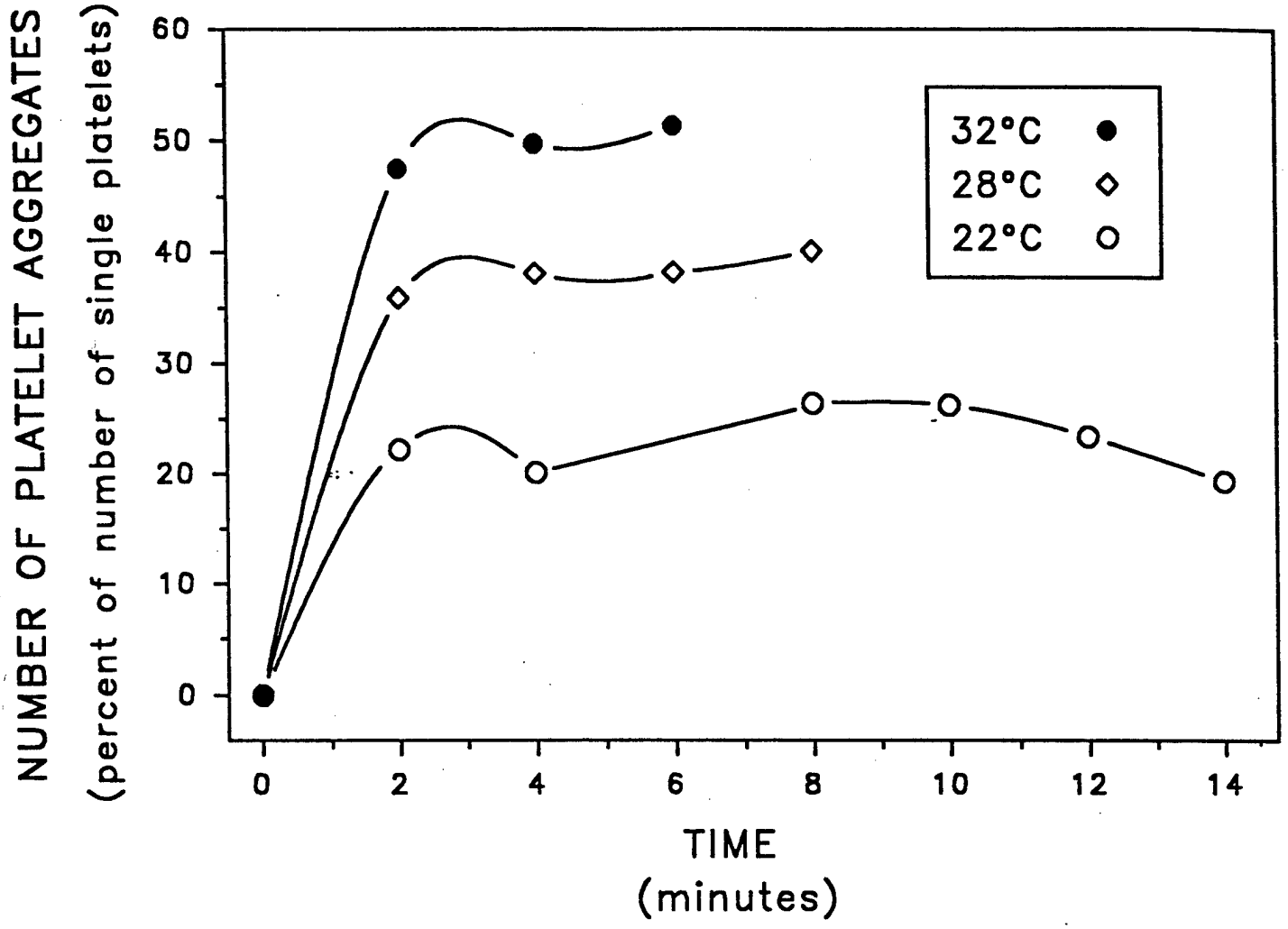
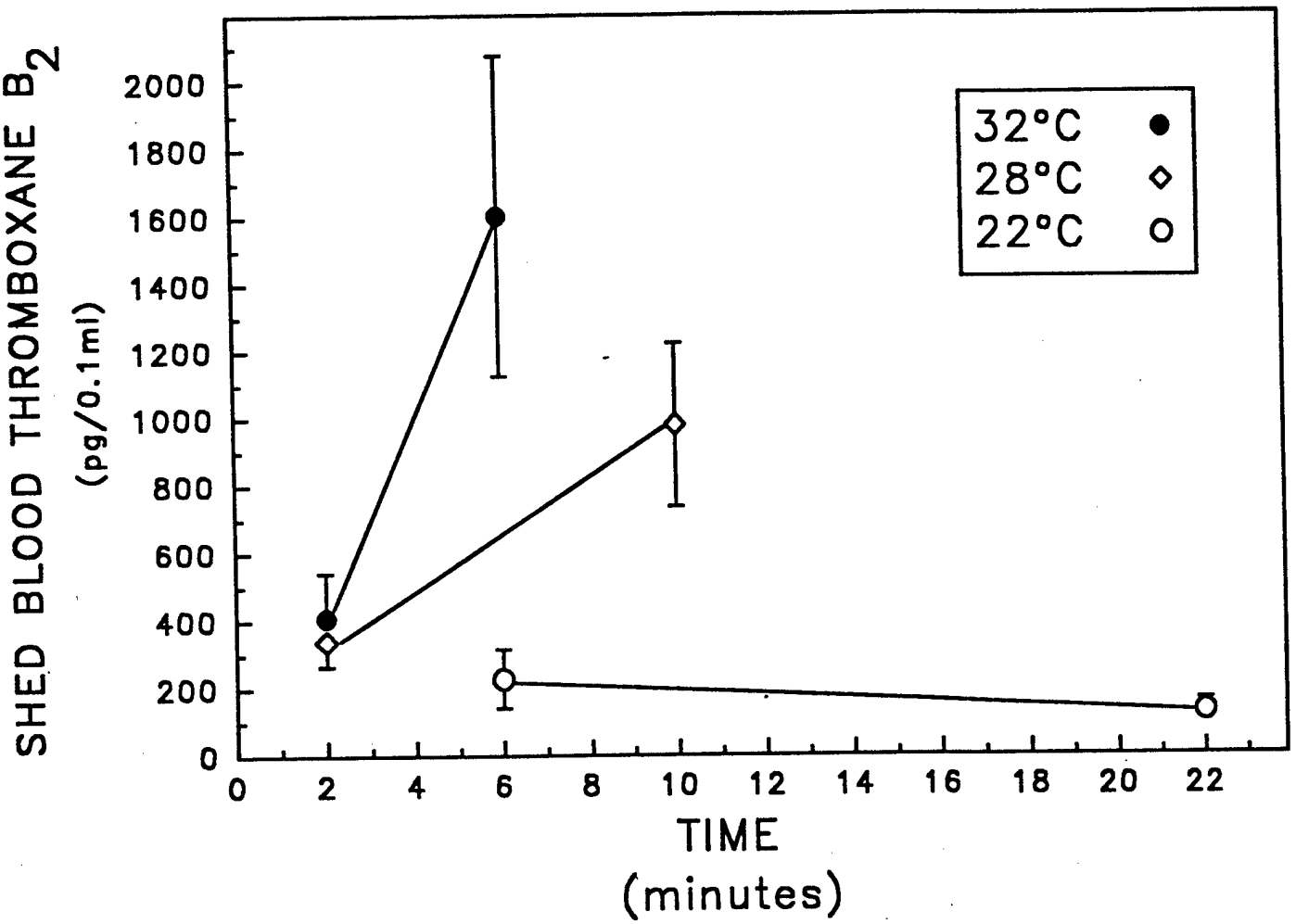

NUMBER OF PLATELET AGGREGATES
(percent of number of single platelets)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
TIME
(minutes)
32°C
28°C
22°C

Fig. 12


SHED BLOOD THROMBOXANE $B_2$
(pg/0.1ml)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
18
20
22
TIME
(minutes)
32°C
28°C
22°C